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Neurotrophic Factors: In Vitro Screening Models of Motor
Neuron Degeneration

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<p>TGFβ-like trophic factors have been shown to be protective in acute neuronal injury paradigms. In the current studies, we analyzed and compared members of this growing family, including GDNF, neurturin, nodal, persephin, TGFβ1 as well as neuroimmunophilin ligand GPI-1046, for protection against chronic glutamate toxicity and the mechanisms for protection. In parallel, we developed a novel organotypic spinal cord culture system to study the ability of these factors to promote motor axon outgrowth. Using these systems, we were able to differentiate the neuroprotective effect of the TGFβ-like factors from their motor axon outgrowth-promoting activity. GDNF, neurturin, persephin, and nodal all protect against excitotoxic motor neuron degeneration. Low amounts of GDNF (1 ng/ml) and high concentrations of neurturin induced vigorous motor axon outgrowth. In contrast, nodal, persephin and TGFβ1 did not induce motor axon outgrowth. Finally, we have ascertained that the neuroprotective properties of some of these compounds (GDNF and GPI-1046) may lie in their ability to induce rapid synthesis of the glutamate transporter EAAT2.</p>		14. SUBJECT TERMS	
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FOREWORD

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N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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Introduction

We will use two *in vitro* models relevant to neurodegeneration to investigate the neuroprotective potential of new TGF trophic factors and new synthetic immunophilin ligands. These agents will be studied in a model of chronic **excitotoxicity** and chronic **oxidative stress** using organotypic spinal cord cultures and in cultures prepared from transgenic mice with human SOD1 mutations. To date, this model has been relatively accurate in predicting agents with clinical efficacy in the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease). Furthermore, these models will enable the detection of new agents capable of inducing rapid motor axon growth.

Body

The original Aims for years 1-3 of the proposal included the following:

Aim 1. Neuroprotection: excitotoxicity. To determine if selected members of the TGF family of neurotrophic factors (neurturin, nodal, and GDNF) or other potent neurotrophic factors (e.g., carditropin-1) or small molecule synthetic factors (e.g. GPI-1046; Gilford) can prevent motor neuron degeneration in a model of chronic excitotoxicity.

- A. To investigate the effects of selected TGF family trophic factors, GPI-1046 and carditropin-1 on motor neuron growth and survival in organotypic spinal cord cultures
- B. To evaluate the neuroprotective properties of selected TGF family trophic factors, GPI-1046 and carditropin on motor neurons in a model of chronic excitotoxicity.
- C. To investigate the mechanisms by which TGF trophic factors protect against excitotoxic motor neuron degeneration: alterations in glutamate receptor subunits that modulate calcium permeability (GluR2), and glutamate transporters.

Significance: These studies could serve as indicators of potent neuroprotectants and motor axon growth stimulation that have the potential to alter the course of ALS and/or repair motor axons after injury.

Aim 2. Neuroprotection: mutant SOD1. To determine if selected members of the TGF β family of neurotrophic factors (neurturin, nodal, and GDNF) or other potent neurotrophic factors (e.g., carditropin-1) or small molecule synthetic factors (e.g. GPI-1046; Gilford) can prevent degeneration of motor neurons cultured from mice transgenic for mutant SOD1.

- A. To investigate the effects of selected TGF β family trophic factors, GPI-1046 and carditropin-1 on altering, *in vitro*, motor neuron degeneration from mice with human SOD1 mutations.
- B. To investigate the mechanisms by which TGF β trophic factors protect against motor neuron degeneration associated with the SOD1 mutations: modulation of

oxidative stress-induction of antioxidant protection systems and/or alterations in glutamate receptor subunits that modulate calcium permeability (GluR2).

Most of Aim 1 projects were addressed in the first year of this grant proposal, recently resulting in a publication¹, and presentations at National meetings².

In Year two of the grant (this years report), the original proposal outlined plans to evaluate aims Aim 1C, Aim 2A, and Aim 2B. Most of the studies in those Aims were completed during the year and some of those results were presented at National meetings²⁻⁶. A manuscript based on that data is now being prepared for peer-review publication.

Neuroprotection in ALS Models and Excitotoxicity

Last year demonstrated that a novel nonimmunosuppressive immunophilin ligand, GPI-1046 was highly protective of motor neurons in an in-vitro model of excitotoxicity. The mechanism of this neuroprotective effect was unknown. In the present study we studied the hypothesis that the neuroprotective properties of GPI-1046 may be due, in part, to up regulation of the glutamate transporter GLT-1.

Excessive accumulation of extracellular glutamate results in neuronal death. Termination of synaptic glutamate transmission and the prevention of excitotoxicity depend on rapid removal of glutamate by high affinity transporters. Of the 5 transporters expressed in the mammalian brain, GLT-1 (EAAT2) is the most abundant and responsible for the bulk of all glutamate transport, accounting for 90% of all transport activity. This transporter plays a crucial role in the prevention of chronic glutamate neurotoxicity, and is markedly decreased in amyotrophic lateral sclerosis (ALS). Recent studies have shown that GLT-1 expression can be induced in-vitro by various factors. The immunophilin ligand GPI-1046 (3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate) is a synthetic, non-immunosuppressive derivative of FK506. In the spinal cord organotypic culture model of chronic excitotoxicity GPI-1046 exerts a potent neuroprotective effect on motor neuron survival. We found that in the same organotypic model GPI-1046 increases GLT-1 immunoreactivity by over 100%, and causes a similar increase in DHK-sensitive Na⁺-dependent glutamate uptake. We further demonstrated induction of GLT-1 expression and function in-vivo, by both, oral-dosing, and intraventricular infusion of rodents with this neuroimmunophilin ligand. We hypothesize that the neuroprotective properties of this agent may be due, in part, to stimulation of glutamate transporter expression and activity, and that such an induction may become a valuable therapeutic approach against excitotoxicity. In a similar fashion we were also able to show that GDNF induced GLT-1 expression by over 100%. We further demonstrated specific inductions in GLT-1 levels in several brain regions of male, Sprague Dawley rats following intraventricular infusion with GDNF.

The glutamate transporter EAAT2 has been shown to be deficient in ALS and antisense knockdown of EAAT2 leads to motor neuron degeneration. As described above, our preliminary data suggested that GPI-1046 may increase expression of

EAAT2 protein and function. We therefore studied the effect of GPI-1046 on survival and motor performance in the G93A transgenic mouse model of familial ALS. Two lines of transgenic mice, a high expresser (G1H+) and a low expresser (G1L+) were used.

G1H/+ transgenic mice received a subcutaneous injection of either 20 mg/kg or 40 mg/kg of body weight of GPI-1046 on 5 days out of 7 from the age of 50 days. G1L/+ transgenic mice received an oral administration of 50 mg/kg of body weight of GPI-1046 twice a day from the age of 150 days. The oral administration of GPI-1046 was able to significantly delay the onset of motor impairment (quantified by failure on rotarod) from 212.2+/-12.47 (n=14) to 234.1+/-10.65 (n=15) days of age (P= 0.048; one tail T-test) and thus extend the survival by approximately 12%. No significant changes were observed compared with the control group in the high expressor line of mice. GPI-1046 may therefore have therapeutic potential in neurological diseases such as ALS, Alzheimer's disease, Parkinson's disease and stroke.

Finally, since glutamate transport may be an important process to minimize glutamate toxicity, and the drugs described above provide one means to modulate this important protein, we have developed a program to identify proteins that interact and modify glutamate transporter activity. We recently completed such a screen and identified two such proteins, GTRAP (glutamate transporter associated proteins) that can potently stimulate neuronal glutamate transporters. This data is included in our recent publication⁷.

Key Research Accomplishments:

- Identification of new TGF β 1 neuroprotective factors
- New methods to prevent motor neuron degeneration
- New agents to enhance motor axon growth
- A new method to screen for drugs/factors capable of enhancing motor nerve growth
- Extended survival in a transgenic mouse model of ALS by the neuroimmunophilin GPI-1046
- Identification of GTRAP41 and GTRAP48- positive modulators of glutamate transport.

Reportable outcomes

1. TGF β -like trophic factors induce synthesis of glutamate transporter proteins. This discovery provides one possible mechanism for action of these agents in their neuroprotective profile discovered in our earlier work¹.

2. Neuroimmunophilin compounds are capable of inducing the synthesis of the predominant glutamate transporter EAAT2. This can occur even with oral delivery.
3. Neuroimmunophilin GPI-1046 can increase survival in an ALS transgenic model.
4. GTRAP41 and GTRAP48 are novel potent stimulators of cerebellar specific glutamate transport protein EAAT4.

Conclusions to date

Members of TGF β -like trophic factors family including GDNF, neurturin, persephin, nodal and TGF β 1 can protect motor neurons against chronic glutamate toxicity. However, only GDNF and neurturin can induce motor axon outgrowth and this effect appears to be mediated through the RET receptor and the MAP kinase/PIK3 pathways. We have now learned that the neuroprotection afforded by these compounds may be due to their ability to induce the synthesis and activation of glutamate transporters. These compounds may be useful as potential therapeutic agents in treating neurodegenerative diseases such as ALS, Parkinson's disease and other disease in which glutamate excess may be a contributing factor. The axon growth stimulation of some of these compounds, as reported last year, may also have relevance in peripheral nerve injuries and spinal cord injury.

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6. Ganel R et al. The neuroimmunophilin ligand GPI-1046 increases glutamate uptake and GLT-1 immunoreactivity in vitro and in vivo. *Society for Neuroscience* **26**,Abstract 539.3(2000)

7. Jackson M., Song W., Liu M.Y., Jin L., Dykes-Hoberg M., Lin C.G., Bowers W.J., Federoff H.J., Sternweis P.C., Rothstein J.D. Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins. *Nature*, 2001, 401:89-93..

Appendix

1. Society for Neuroscience Abstracts- 1999
2. Society for Neuroscience Abstracts- 2000
3. Jackson M., Song W., Liu M.Y., Jin L., Dykes-Hoberg M., Lin C.G., Bowers W.J., Federoff H.J., Sternweis P.C., Rothstein J.D. Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins. *Nature*, 2001, 401:89-93.

SOCIETY FOR NEUROSCIENCE

ABSTRACTS

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539.1

NIFLUMIC ACID MODULATES PROTON AND CHLORIDE CONDUCTANCES OF THE GLUTAMATE TRANSPORTER EAAT4. R.J. Vandenberg¹, M.V. Poulsen. *Dept Pharmacol, Univ Sydney, Sydney, Australia*

The glutamate transporter EAAT4 exhibits several ionic currents that are either coupled or uncoupled to the transport of substrate. Drugs that can modulate the function of the transporter are important both as a means to understand the pharmacology and physiology of the transporter as well as a lead in the development of novel therapeutics. The EAAT4 transporter was expressed in *Xenopus* oocytes and two electrode voltage clamp techniques used to measure transport currents. 300 μ M Niflumic acid, a non-steroidal anti-inflammatory drug and chloride channel blocker, caused a 2 fold increase in the current elicited by 10 μ M L-aspartate, but does not affect the rate of 3H-L-aspartate uptake or the apparent affinity of L-aspartate. Co-application of the glutamate transport blocker DL-threo-benzylxylaspartate with L-aspartate and niflumic acid blocked both the L-aspartate induced current and the current elicited by niflumic acid, which indicates that both currents are mediated by EAAT4. Current voltage relationships measured with different extracellular proton and chloride concentrations demonstrated that the current is mediated by both chloride and protons. Arachidonic acid has been shown to elicit a proton conductance in EAAT4 and so we investigated whether niflumic acid and arachidonic acid compete for the same site on the transporter. Application of a maximal dose of arachidonic acid stimulated the L-aspartate activated conductance 2 fold and subsequent co-application of niflumic acid caused a further 2.5 fold increase in the current. This suggests that arachidonic acid and niflumic acid do not compete for the same site, but do have similar functional consequences. *Supported by: Australian NHMRC.*

539.3

THE NEUROIMMUNOPHILIN LIGAND GPI-1046 INCREASES GLUTAMATE UPTAKE AND GLT-1 IMMUNOREACTIVITY IN VITRO AND IN VIVO. R. Ganell^{1,2}, C. Coccia^{1,2}, J. Steiner^{1,2}, J. Rothstein^{1,2}. ¹*Department of Neurology and Neuroscience, Johns Hopkins University, Baltimore, MD, USA*, ²*Guilford Pharmaceuticals Inc., Baltimore, MD, USA*

Excessive accumulation of extracellular glutamate results in neuronal death. Termination of synaptic glutamate transmission and the prevention of excitotoxicity depend on rapid removal of glutamate by high affinity transporters. Of the 5 transporters expressed in the mammalian brain, GLT-1 (EAAT2) is the most abundant and responsible for the bulk of all glutamate transport, accounting for ~90% of all transport activity. This transporter plays a crucial role in the prevention of chronic glutamate neurotoxicity, and is markedly decreased in amyotrophic lateral sclerosis (ALS). Recent studies have shown that GLT-1 expression can be induced in-vitro by various factors. The immunophilin ligand GPI-1046 is a synthetic, non-immunosuppressive derivative of FK506. It was found to delay the onset of neuronal deficits in SOD-1 transgenic mice (ALS model). In the spinal cord organotypic culture model of chronic excitotoxicity GPI-1046 exerts a potent neuro-protective effect on motor neuron survival. We found that in the same organotypic model GPI-1046 increases GLT-1 immunoreactivity by over 100%, and causes a similar increase in DHK-sensitive Na^+ -dependent glutamate uptake. We further demonstrated induction of GLT-1 expression and function in-vivo, by both, oral-dosing, and intraventricular infusion of rodents with this neuroimmunophilin ligand. We hypothesize that the neuroprotective properties of this agent may be due, in part, to stimulation of glutamate transporter expression and activity, and that such an induction may become a valuable therapeutic approach against excitotoxicity. *Supported by: NIH (NS33958, NS36465) and MDA.*

539.5

THE TRANSIENT EXPRESSION OF THE GLUTAMATE TRANSPORTERS GLAST AND GLT-1 IN HIPPOCAMPAL NEURONS IN CULTURE IS DEPENDENT OF DIFFUSIBLE GLIAL FACTORS AND NEURONAL GLIAL CONTACTS, RESPECTIVELY. C. Plâcher¹, S. Gaillet¹, N. Danbolt², M. Récasens^{1*}. ¹*UMR 5102 CNRS, Univ. Montp 2, Montpellier, France*, ²*Anat Inst., Univ Oslo, Oslo, Norway*

We have shown by immunocytochemistry that hippocampal neurons grown in a defined medium not only expressed the neuronal EAAC glutamate transporter, but also the <> glial >> glutamate transporters GLAST and GLT-1 at early stage of the culture (from 1 to 8 day in vitro). Then, as far as neurons formed synapses and the scarce glial cells, present in this type of culture, tremendously developed their extensions, this abnormal expression disappeared, we investigated whether glial cells could be responsible for the disappearance of GLAST and GLT-1 nominal expression. We found that i) conditioned medium from pure mature astrocytic culture rapidly inhibited the GLAST expression in neurons, without affecting that of GLT and ii) culturing neurons on a mature glial cell layer suppressed the expression of both GLAST and GLT in neurons. These data provide the first evidence that GLAST and GLT expression in neurons is under the control of glia. This control is assumed either by released factors (for GLAST) or by glia-neuron contacts (GLT). More generally, this suggests that glial cells could influence the membrane phenotype of neurons.

539.2

ABOLITION OF QUISQUALATE SENSITIZATION AND ENHANCEMENT OF POTENCY FOR DEPOLARIZATION BY QUISQUALATE IN 24-HOUR HIPPOCAMPAL SLICES BRIEFLY EXPOSED TO QUISQUALATE. J.F. Koerner¹, A.J. Beitz, L.L. Wellman, L.A.

Chase. *Depts. of Biochem., Mol. Biol. & Biophys. and Veterinary Pathobiol, University of Minnesota, Minneapolis, MN, USA*

Quisqualic acid (QUIS) sensitizes CA1 pyramidal neurons in rat hippocampal slices to depolarization by glutamate analogues including L-2-amino-6-phosphonohexanoic acid (L-AP6) and L-AP4 (Quisqualate Sensitization; QUIS-effect). The QUIS is concentrated in interneurons by a Na^+ -independent glutamate-cystine antiporter, System x_c^- , and is released when interneurons are exposed to L-AP6. Rat hippocampal slices were incubated for 24 hrs at 34° C in bicarbonate-buffered medium including Ca^{2+} (2.5 mM), Mg^{2+} (2.4 mM) & glucose (10 mM). These slices continued to exhibit strong stimulus-evoked antidromic population spikes in the CA1 region and also Schaffer collateral-CA1 pyramidal cell extracellular field potentials. Slices exposed to 16 μ M L-QUIS for 4 min 1 hr after preparation and then incubated overnight also exhibited antidromic population spikes but were not depolarized by exposure to 200 μ M L-AP6. Thus the QUIS-effect, which can be repeatedly evoked by exposure to L-AP6 on the first day, was no longer observed. It could not be restored by again exposing the slice to L-QUIS after overnight incubation. Also the IC_{50} for depolarization of the CA1 pyramidal cells by QUIS was 0.3 μ M, in contrast to the IC_{50} of 10 μ M for depolarization of fresh slices or 24-hr slices not previously exposed to QUIS. This IC_{50} (0.3 μ M) was the same as that observed for depolarization by QUIS in the presence of 400 μ M L-cystine, suggesting that abolition of Na^+ -independent transport is related to this phenomenon. *Supported by: NIH NS 35073.*

539.4

FUNCTIONAL INDUCTION OF THE L-GLUTAMATE TRANSPORTER IN MAMMALIAN GLIAL CELLS BY THE PRESENCE OF NEURONES OR NANOMOLAR CONCENTRATIONS OF L-GLUTAMATE. C.L. Yamate^{1*}, D. Bertrand¹, T. Rauer², L. Vutskits¹. ¹*Dept. of Physiology and Morphology, Univ. Geneva, Geneva 4, Switzerland*, ²*Max-Planck Institute for Brain Research, Frankfurt, Germany*

Neurotransmitter levels of L-glutamate (L-glu) and maintenance of glutamatergic synaptic transmission are believed to be linked to the expression and regulation of the Na^+ -dependent electrogenic L-glu transporter in glia. To test this assumption, the functionality of the L-glu transporter was examined in rat cortical astrocytes using the pH fluorescence indicator, BCECF. A spatially restricted subpopulation of astrocytes on neurone-glia microislands responded to L-glu (200 μ M) by a fast and transient acidification. No detectable change in the pH could be observed, however, when astrocytes were grown alone, although they expressed a normal level of plasma membrane transporter proteins (GLAST). Based on this observation we speculated that when cultured in isolation, astroglia express a significant amount of the L-glu transporter, but that due to lack of substrate, these proteins remain in a non-functional state. This hypothesis was confirmed by pre-exposure to L-glu (4H, 0.1-100 μ M) which caused a concentration-dependent increase in acidification starting at <100nM L-glu. These data indicate that when grown together, neurones and glia interact and maintain the glutamate transporter in a functional state, a condition that can be mimicked by the addition of exogenous L-glutamate. *Supported by: This work was supported by the Swiss National Science Foundation grant to C.L.Y.*

539.6

ALTERATION IN DOPAMINE TRANSPORTER KINETICS AFTER PERINATAL LEAD EXPOSURE IN ADULT MALE AND FEMALE RATS. J.B. Alfaro^{1*}, B.A. Sorg⁴, E.K. Silbergeld⁵, J.O. Schenk^{1,2}. ¹*School of Molecular Biosciences*, ²*Department of Chemistry*, ³*Programs in Pharmacology/Toxicology and Neuroscience*, ⁴*Department VCAAPP, Washington State University, Pullman, WA, USA*, ⁵*Program in Human Health and the Environment, Univ. Maryland Med. School, Baltimore, MD, USA*

The mesolimbic dopaminergic system has been previously found to be sensitive to lead exposure. This study examined the effect of perinatal lead (25 ppm) exposure on dopamine transporter function in the nucleus accumbens. Using the technique of Rotating Disk Electrode Voltammetry (RDE), Km and Vmax were measured using dopamine in control and lead-treated offspring in male and female adult rats. In the adult male rats, the control males had significantly higher Km (Control 0.49 μ M, Lead 0.28 μ M) and Vmax (Control 388 pmol/sec/g wet weight, Lead 344 pmol/sec/g wet weight) values than the lead male rats. In the female rats, there was a significant difference in the Vmax value (Control 349 pmol/sec/g wet weight, Lead 261 pmol/sec/g wet weight), but there was no change in Km between the two treatment groups. In both sexes, studies were conducted to examine the effect of perinatal lead on dopamine transporter inhibition by cocaine. There was no significant difference in the K_i between adult control and lead treated males. In the adult female rats, there was a significant difference in the percent of dopamine uptake inhibition under the same conditions (Control 50%, Lead 30%). This data supports previous studies that suggest alterations in the mesolimbic dopaminergic system by lead exposure. *Supported by: JOS grants from the State of Washington.*

SOCIETY FOR NEUROSCIENCE

ABSTRACTS

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638.5

GPI-1046, A NOVEL NON-IMMUNOSUPPRESSANT IMMUNOPHILIN LIGAND, DELAYS THE APPEARANCE OF MOTOR DEFICITS IN A TRANSGENIC MOUSE MODEL OF FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS. J.D. Rothstein¹, M. Jackson¹, C. Sakai¹, D.M. Spicer², B.D. Kim¹ and J.P. Steiner¹. Johns Hopkins University, Department of Neurology and Neuroscience, Baltimore MD, 21287, ²Guilford Pharmaceuticals Inc, Baltimore MD, 21224.

GPI-1046 (3-(3-pyridyl)-1-propyl(2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyridinecarboxylate), a synthetic non-immunosuppressive immunophilin ligand, has previously been shown to be neurotrophic in multiple neuronal systems, be neuroprotective in the organotypic spinal cord culture model of chronic excitotoxicity and has recently been shown to induce the expression of GLT-1 (EAAT2), the glutamate transporter markedly decreased in amyotrophic lateral sclerosis (ALS). We therefore studied the effect of GPI-1046 on survival and motor performance in the G93A transgenic mouse model of familial ALS. Two lines of transgenic mice, a high expressor (G1 H⁺) and a low expressor (G1 L⁺) were used.

G1 H⁺ transgenic mice received a subcutaneous injection of either 20 mg/kg or 40 mg/kg of body weight of GPI-1046 on 5 days out of 7 from the age of 50 days. G1 L⁺ transgenic mice received an oral administration of 50 mg/kg of body weight of GPI-1046 twice a day from the age of 150 days. The oral administration of GPI-1046 was able to significantly delay the onset of motor impairment (quantified by failure on rotarod) from 212.2 ± 12.47 (n=14) to 234.1 ± 10.65 (n=15) days of age (P=0.048; one tail T-test) and thus extend the survival by approximately 12%. No significant changes were observed compared with the control group in the high expressor line of mice.

GPI-1046 may therefore have therapeutic potential in neurological diseases such as ALS, Alzheimer's disease, Parkinson's disease and stroke. Supported by NIH (NS33958), Muscular Dystrophy Association and Project A.L.S.

638.7

THE MICRONESIAN HEALTH STUDY BRAIN BANK: A RESOURCE FOR RESEARCH ON GUAM AMYOTROPHIC LATERAL SCLEROSIS-PARKINSONISM-DEMENTIA COMPLEX (ALS/PDC). D.P. Perl¹, B.E. Good¹, U.K. Craig², P.R. Hof², D. Galasko⁴, W.C. Wiederholt⁴. ¹Dept. of Pathology, and ²Neurobiology of Aging Laboratories, Mount Sinai School of Medicine, New York, NY 10029, ³Dept. of Nursing and Health Policy, Univ. of Guam, Mangilao, Guam, ⁴Dept. of Neuroscience, Univ. of California San Diego, La Jolla, CA

Since the initial report of Zimmerman 1944, ALS/PDC, an endemic disorder of the indigenous Chamorro people of Guam has been the subject of intense research interest and investigation. The disorder manifests itself as either a)ALS, clinically indistinguishable from the sporadic form seen elsewhere in the world, or b) PDC, a disorder with parkinsonian features including rigidity and bradykinesia, accompanied by profound dementia. Overlap between the two forms is quite common. In addition, a purely dementing form of the illness is encountered with progressive dementia in the absence of amyotrophy and parkinsonian features. The brains of affected patients show evidence of upper and lower motor neuron degeneration (in ALS cases), or substantia nigra neuronal loss (in PDC cases), both accompanied by widespread severe neurofibrillary tangle (NFT) formation. Based on ultrastructural, immunohistochemical and molecular biologic studies, these NFTs are identical to those of Alzheimer's disease, yet are encountered in the absence of significant beta-amyloid accumulation in the form of senile plaques. This unique disorder represents an important model for the study of many aspects of the age-related neurodegenerative disorders. Despite reports to the contrary, large numbers of affected individuals are still encountered on Guam. The Micronesia Health Study is an NIH-funded study (PO1 AG-14382) of many aspects of ALS/PDC. In this project we have established a tissue repository of well-characterized brain tissue specimens derived from ALS-PDC cases and Guam controls. Formalin fixed and paraffin-embedded tissues are available along with a modest amount of freshly obtained tissues which are frozen at -80°C. Selected tissues have undergone non-biased sampling in preparation for stereologically based studies. We wish to make these tissues widely available to qualified scientists pursuing meaningful research questions. The nature of the collection and method for applying for specimens will be provided at the poster or can be obtained from Dr. Wiederholt at wwiederholt@ucsd.edu.

638.9

GDNF AND BDNF IN CEREBROSPINAL FLUID FROM PATIENTS WITH MOTOR NEURON DISEASE. E. Grundström^{1,*}, H. Askmark¹, L. Korhonen², D. Lindholm² and S.-M. Aquilonius¹. Department of Neuroscience, ¹Neurology, University Hospital, S-751 85 Uppsala, Sweden and ²Developmental Neuroscience, Biomedical Center, Box 587, S-751 23 Uppsala, Sweden.

Growth factors such as GDNF, BDNF, CNTF and IGF-1 have been shown to rescue motor neurons both in vitro and in vivo in animal models. In degenerative diseases as ALS (amyotrophic lateral sclerosis) GDNF has been shown to be increased in muscle tissue and it is possible that these alterations in growth factor expression can be depicted also in CSF (cerebrospinal fluid) samples. In the present study we compare levels of GDNF and BDNF in CSF in a group of ALS patients, patients with other neurological diseases and healthy controls.

The CSF is collected and stored at -70 °C prior to analysis by ELISA technique.

Preliminary data show no increase in GDNF levels in ALS patients compared to controls in contrast to the increased expression of GDNF mRNA previously observed in ALS skeletal muscle. Studies on BDNF levels in the same population is ongoing.

Supported by the Swedish Medical Research Council (project 4373).

638.6

MOTOR NEURON DISEASE WITH NEUROFIBRILLARY TANGLE (MNNDFT): A NEW SUBTYPE OF AMYOTROPHIC LATERAL SCLEROSIS (ALS) WITH DEMENTIA (ALSD). Y. Saito^{1,2}, Y. Motoyoshi³, M. Kunimoto⁴, Y. Tashiro⁵ and S. Murayama¹. ¹Dept. of Neuropathology, Tokyo Metropolitan Institute of Gerontology; ²Dept. of Neurol. Univ. Tokyo; ³Dept. of Neurol., National Shimoshizu Hosp.; ⁴Dept. of Neurol. & ⁵Pathol, Yokohama Rosai Hosp., Tokyo, Japan, 173-0015

Two late onset cases of amyotrophic lateral sclerosis (ALS) in, 84 (case 1) and 80 year old (case 2) males, were reported. Both presented with stubbornness and difficulty in obeying each medical instruction unless completely persuaded. Both died of respiratory failure. Total clinical course was 8 months, and 4 years and 4 months respectively. Postmortem examination confirmed typical pathology of ALS. In addition, many NFTs with ghost tangles were preferentially detected in the CA2 region of the hippocampus. The NFTs were immuno-reactive with various antibodies against phosphorylation-dependent and -independent form of τ , as well as anti-ubiquitin antibodies, and composed of paired helical filaments. A few diffuse plaques were recognized in case 2 with anti- β antibodies but no neuritic plaques were identified. The CA2 region was usually relatively spared from NFT pathology in normal aging. Thus, the preferential involvement of CA2 with NFT in these cases could contribute to the unique character change and may form a cardinal pathology of this form of ALS. The etiopathological factor common to classic pathology of ALS and localized appearance of NFT in CA2 remains to be clarified.

638.8

EFFECTS OF CELLULARLY DERIVED GDNF TREATMENT IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS. M.K. Housewright¹, T.L. Williamson¹, A. Blesch², M.H. Tuszynski², D.W. Cleveland^{1,2*}. ¹Ludwig Institute for Cancer Research, ²Department of Neuroscience, University of CA, San Diego 92093.

Neurotrophic factors such as glial cell line derived neurotrophic factor (GDNF) have been shown to promote the growth and survival of motor neurons. In several experimental models, GDNF is more potent than CNTF, NGF, BDNF, and IGF-1 for motor neuron survival, fostering expectations that it would be useful in treating human motor neuron diseases such as amyotrophic lateral sclerosis (ALS). In order to test whether administration of GDNF could prevent the death of motor neurons in ALS, fibroblasts harboring the Moloney murine leukemia virus expressing this neurotrophic factor were injected into the gastrocnemius muscles of SOD-1 mutant mice. These mutant mice are well established as a model of familial ALS and exhibit pathology, muscle wasting, and paralysis similar to that observed in human ALS patients. The efficacy of this therapeutic strategy was tested by a number of morphological and functional analyses. The motor performance, age of disease onset and age of death for mice injected with GDNF producing cells was compared with that of mice receiving cells expressing GFP or that of mice receiving no injection. Preliminary results indicate that functional improvements were not realized and no alteration in lifespan was achieved. Funded by an NIH grant.

638.10

NEUROFILAMENT SIDE-ARMS ARE PHOSPHORYLATED BY MULTIPLE KINASES AND SIDE-ARM PHOSPHORYLATION INFLUENCES THE PROPERTIES OF NEUROFILAMENTS. C.C.J. Miller¹, J. Browne², S. Ackery¹, A.J. Grierson¹, P. Thornhill¹, A. Yates¹, C.E. Shaw¹. Departments of Neuroscience and Clinical Neurosciences, The Institute of Psychiatry, De Crespigny Park, Denmark Hill, London SE5 8AF UK.

Neurofilaments are the major intermediate filaments of neurones and neurofilament proteins are phosphorylated. Using a combination of in vitro, cell transfection and mutagenic studies, we have investigated the mechanisms that regulate neurofilament heavy chain (NF-H) side-arm phosphorylation. A variety of kinases phosphorylate NF-H side-arms and these include cyclin dependent kinase-5 (cdk5), glycogen synthase kinase-3 (GSK-3 α / β) and members of the mitogen-activated protein kinase (MAPK) family. At least some of these kinases target different or overlapping sites in NF-H side-arms. The signal transduction pathways that regulate activities of these kinases have been investigated and extracellular signals have been shown to alter NF-H phosphorylation. The effects that changes in neurofilament phosphorylation have on the cellular properties of neurofilaments are being studied using green fluorescent protein-tagged neurofilament proteins. Abnormal accumulations of phosphorylated neurofilaments are a pathological feature of motor neurone disease (amyotrophic lateral sclerosis). A better understanding of the molecular mechanisms that regulate neurofilament phosphorylation therefore has relevance to MND.

Supported by grants from the Wellcome Trust and UK Motor Neurone Disease Association to CCJM.

169.7

Serine/threonine phosphatase activity of calcineurin is inhibited by sodium orthovanadate and dithiothreitol reverses the inhibitory effect. T. Kawano¹, M. Morioka¹, K. Fukunaga², S. Hasegawa¹, K. Korematsu¹, Y. Kai¹, I. Hamada¹, E. Miyamoto², Y. Ushio¹. Dept. of Neurosurgery and ²Pharmacology, Kumamoto Univ. Med. Sch.; Kumamoto 860-8556, Japan

Orthovanadate is known to be an inhibitor of protein tyrosine phosphatases. However, we found that it inhibited calcineurin which has the activity of a serine/threonine protein phosphatase, using casein phosphorylated by cyclic AMP-dependent protein kinase as a substrate. Orthovanadate inhibits the Mn²⁺-activated activity of purified calcineurin to 20%, this is not the case without Mn²⁺. Furthermore, 10 mM dithiothreitol (DTT) reversed the inhibitory effects of orthovanadate. Orthovanadate showed the same inhibitory effect for calcineurin activity in hippocampal homogenates as for the purified enzyme; the inhibitory effect was reversed by DTT. These results indicate that orthovanadate inhibits not only protein tyrosine phosphatases as reported, but also serine/threonine phosphatase activity of calcineurin.

169.8

CHARACTERIZATION OF A SPINOPHILIN/p70 rsk COMPLEX.

P. B. Allen¹, P. E. Bennett², S. H. Snyder², and P. Greengard¹. ¹Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY 10021. ²Department of Neuroscience, The Johns Hopkins School of Medicine, Baltimore, MD 21025.

The postsynaptic density contains many of the enzymes known to be involved in the regulation of synaptic transmission. Protein phosphatase 1 is required for the regulation of long-term changes in synaptic strength, and is thought to be targeted to dendritic spines through its interaction with the cytoskeletal protein spinophilin. Structurally, spinophilin is closely related to neurabin, a neuronal F-actin binding protein. Neurabin has also been characterized as a binding partner for p70 rsk. This interaction, mediated by the COOH-terminus of p70 rsk and the neurabin PDZ domain, results in the modulation of p70 rsk activity. We have examined the ability of p70 rsk to interact with spinophilin; we detect binding by both yeast 2-hybrid and co-precipitation assays. In addition, a synthetic peptide that mimics the COOH-terminus of p70 rsk reduces the spinophilin-mediated modulation of protein phosphatase 1 activity. These results suggest that phosphatase regulation is dependent upon the macromolecular context. This may reflect a requirement for dynamic regulation of these enzyme-cytoskeleton interactions at the synapse.

This work was supported by U.S. Public Health Service Grant #MH40899.

169.9

IDENTIFICATION AND STRUCTURAL ANALYSIS OF A NEURABIN AND SPINOPHILIN COMPLEX. P.E. Bennett¹, M.M. Lai¹, P.B. Allen², P. Greengard² and S.H. Snyder¹. ¹Dept. of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD 21205; ²Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY 10021.

Neurabin and spinophilin are homologous F-actin binding proteins found in neurons. Spinophilin has been localized to spines by electron microscopy and has been implicated in the modulation of neostriatal AMPA channel activity by specifically binding to protein phosphatase-1 (PP-1). Neurabin, a neuron specific protein, is required for neurite outgrowth in cultures of rat hippocampal neurons. Via its PDZ domain, neurabin binds and stimulates the mitogen activated protein kinase p70. Spinophilin and neurabin are both capable of binding and bundling F-actin.

Unlike the neuronally restricted neurabin, spinophilin is expressed in many different tissue types and is found at cadherin-based cell-cell adhesion sites. However, these two proteins have a significant overlap of subcellular localization in neurons. Here we demonstrate a complex of neurabin and spinophilin using both *in vivo* and *in vitro* methods. Deletional analyses of neurabin and spinophilin are consistent with the carboxy-terminal regions mediating the formation of this complex. This region is rich in coiled-coil domains in both proteins and has been hypothesized to mediate the previously observed homotypic (neurabin-neurabin or spinophilin-spinophilin) interactions.

Preliminary results suggest that the coiled-coil domain is required for both homotypic and heterotypic (neurabin-spinophilin) complex formation as well as actin bundling.

TRANSPORTERS: EAA I

170.1

THE NEUROIMMUNOPHILIN LIGAND GPI-1046 INDUCES THE EXPRESSION OF GLUTAMATE TRANSPORTER OF EAAT2 (GLT-1) SUBTYPE. R. Ganet¹, C. Coccia¹, J. Steiner¹, J.D. Rothstein¹, Johns Hopkins University, Department of Neurology and Neuroscience, Baltimore MD, 21287, Guilford Pharmaceuticals Inc., Baltimore MD, 21224.

Excessive accumulation of extracellular glutamate results in neuronal death. Termination of synaptic glutamate transmission and the prevention of excitotoxicity depend on rapid removal of glutamate by high affinity transporters. Of the 5 transporters expressed in the mammalian brain, EAAT2 (GLT-1) is the most abundant and responsible for the bulk of all glutamate transport, accounting for ~90% of all transport activity. EAAT2 (GLT-1) plays a crucial role in the prevention of chronic glutamate neurotoxicity, and is markedly decreased in amyotrophic lateral sclerosis (ALS). Recent studies have shown that GLT-1 expression can be induced *in-vitro* by various factors.

The immunophilin ligand GPI-1046 (3-(3-pyridyl)-1-propyl (2S)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinecarboxylate) is a synthetic, nonimmunosuppressive derivative of FK506. It was found to delay the onset of neuronal deficits in SOD-1 transgenic mice (ALS model). In the spinal cord organotypic culture model of chronic excitotoxicity GPI-1046 exerts a potent neuroprotective effect on motor neuron survival.

We found that in the same organotypic model GPI-1046 induces the expression of GLT-1 by over 100%. We further demonstrated induction of GLT-1 expression *in-vivo*, by both, oral-dosing, and intraventricular infusion of rodents with this immunophilin ligand.

We hypothesize that the neuroprotective properties of this agent may be due, in part, to stimulation of glutamate transporter activity, and that induction of GLT-1 expression and/or activity may become a valuable therapeutic approach against excitotoxicity. [Supported by NIH (NS33958, NS36465) and MDA].

170.2

GDNF INDUCES AN INCREASE IN EAAT2 (GLT-1)

EXPRESSION. C. Coccia¹, R. Ganet and J.D. Rothstein, Johns Hopkins University, Department of Neurology and Neuroscience, Baltimore MD, 21287.

Termination of synaptic glutamate transmission and the prevention of excitotoxicity depend on rapid removal of glutamate by high affinity transporters. The astroglial transporter EAAT2 (GLT-1) is the most abundant subtype expressed in the mammalian brain. This transporter is responsible for the bulk of all glutamate transport, accounting for ~90% of all transport activity, and was shown to play a crucial role in the prevention of chronic excitotoxicity. Recent studies have shown that GLT-1 expression is regulated by neurons, probably, via diffusible factors. Subsequent studies demonstrated that GLT-1 expression can be induced in cultured astrocytes by various factors.

The organotypic spinal cord culture model was developed in our lab to study chronic excitotoxicity in an environment where spinal synaptic connectivity is maintained and normal astroglia-neuron interactions are preserved. Previously, we showed in this system that factors of the GDNF family produce a potent neuroprotective effect on motor neuron survival under chronic excitotoxicity. We now show that in the same system, factors of the GDNF family induce GLT-1 expression by over 100%. We further demonstrate specific inductions in GLT-1 levels in several brain regions of male, Sprague Dawley rats following intraventricular infusion with GDNF.

We hypothesize that the neuroprotective properties of these factors may be due, in part, to stimulation of glutamate transporter activity, and that induction of GLT-1 expression and/or activity may become a valuable therapeutic approach against excitotoxicity.

[Supported by NIH (NS33958, NS36465) and MDA.]

Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins

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Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system and is removed from the synaptic cleft by sodium-dependent glutamate transporters. To date, five distinct glutamate transporters have been cloned from animal and human tissue: GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5 (refs 1–5). GLAST and GLT-1 are localized primarily in astrocytes^{6,7}, whereas EAAC1 (refs 8, 9), EAAT4 (refs 9–11) and EAAT5 (ref. 5) are neuronal. Studies of EAAT4 and EAAC1 indicate an extrasynaptic localization on perisynaptic membranes that are near release sites^{8–10}. This localization facilitates rapid glutamate binding, and may have a role in shaping the amplitude of postsynaptic responses in densely packed cerebellar

terminals^{12–15}. We have used a yeast two-hybrid screen to identify interacting proteins that may be involved in regulating EAAT4—the glutamate transporter expressed predominately in the cerebellum—or in targeting and/or anchoring or clustering the transporter to the target site. Here we report the identification and characterization of two proteins, GTRAP41 and GTRAP48 (for glutamate transporter EAAT4 associated protein) that specifically interact with the intracellular carboxy-terminal domain of EAAT4 and modulate its glutamate transport activity.

To identify proteins that interact with the C terminus of the EAAT4 protein, we used the last 77 amino acids of EAAT4 as bait to screen a rat brain complementary DNA library. We isolated two independent cDNA clones and called them GTRAP41 and GTRAP48 (for glutamate transporter-4-associated protein). Isolation of the full-length cDNAs by a series of 5' and 3' rapid amplification of cDNA ends (RACE) polymerase chain reactions (PCRs) showed that the largest open reading frame (ORF) for GTRAP41 is 7,164 base pairs (bp), which encodes a 2,388 amino-acid protein with a predicted relative molecular mass (M_r) of 270,958 (accession no. AF225960).

A BLAST search of the GenBank database showed that GTRAP41 possesses 87% identity with β -spectrin III (accession no. AB008567). GTRAP41 possesses seventeen 16-amino-acid spectrin repeats, two α -actinin domains and a pleckstrin homology (PH) domain (Fig. 1a). The largest ORF identified for GTRAP48 (accession no. AF225961) is 4,581 bp, which encodes a 1,527-amino-acid protein with a predicted M_r of 168,698. A BLAST search of the GenBank database showed that GTRAP48 is unique, but it possesses significant homology to the KIAA0380 cDNA encoded protein (90% identity) and RhoGEF, p115 (ref. 16). GTRAP48 possesses a PDZ domain, a regulatory G-protein-signalling sequence (LH),

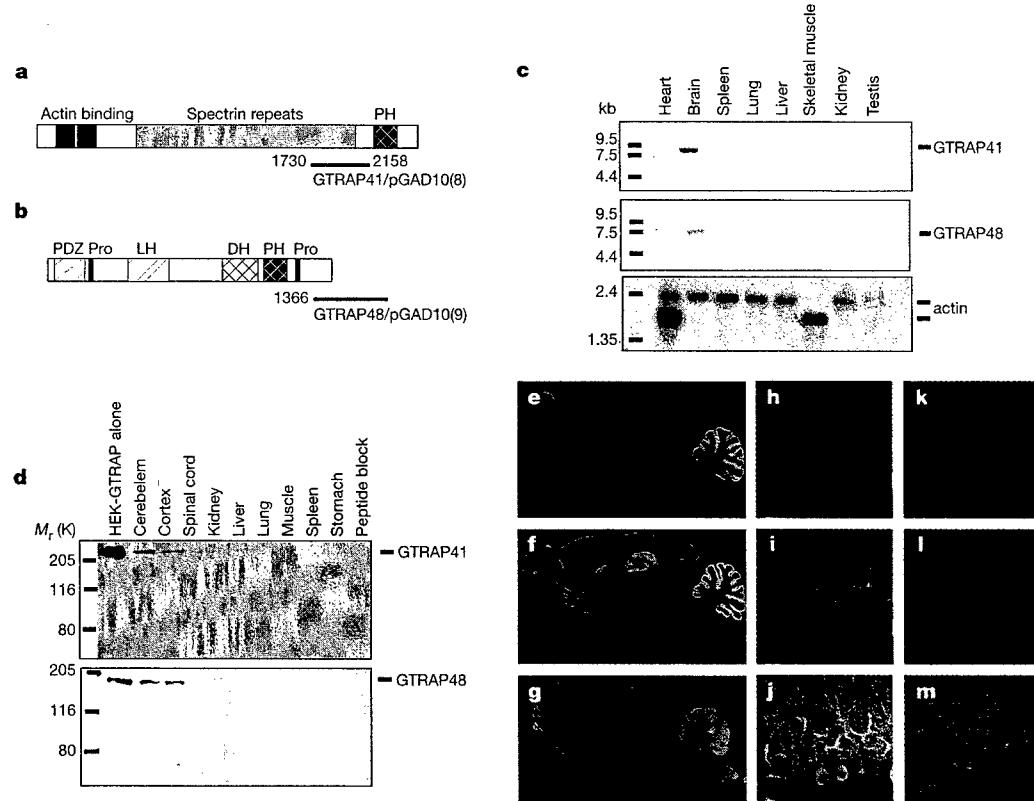


Figure 1 Structure and distribution of GTRAP41 and GTRAP48. **a, b**, cDNA clones GTRAP41/pGAD10 and GTRAP48/pGAD10 isolated from the yeast two-hybrid screen are shown aligned below representations of full-length GTRAP41 and GTRAP48, respectively. The number of times the clones were isolated is shown in parentheses. **c**, Multiple tissue northern (MTN; Clontech) blot probed with 3' PCR probes of GTRAP41 and GTRAP48.

d, Western analysis of GTRAP41 and GTRAP48. **e–g**, Rat brain sections stained with anti-EAAT4 (**e**), anti-GTRAP41 (**f**) and anti-GTRAP48 (**g**) antibodies. **h–m**, EAAT4 (**h**, **k**), GTRAP41 (**i**) and GTRAP48 (**l**) are all predominately expressed in the cell bodies and dendrites of Purkinje cells. The overlaps of GTRAP41 and GTRAP48 with EAAT4 are shown in **j** and **m**, respectively.

tandem dbl homology (DH) and pleckstrin homology (PH) domains characteristic of guanine nucleotide exchange factors for the Rho family of G proteins, and two proline-rich sequences (Fig. 1b).

Northern blot analysis detected a 8.3-kilobase (kb) GTRAP41 and a 7.5-kb GTRAP48 messenger RNA in brain tissue (Fig. 1c). Longer exposure revealed a low level of expression in liver and kidney for both GTRAP41 and GTRAP48. Anti-peptide antibodies were raised and the affinity-purified antibodies recognized a M_r 270,000 (270K) protein and a 170K protein, respectively, in HEK 293T cells transfected with full-length GTRAP41 and GTRAP48 cDNA (Fig. 1d). GTRAP41 and GTRAP48 were both selectively localized to brain, consistent with the northern blot analysis (Fig. 1d). EAAT4 is selectively localized to cerebellar Purkinje cells, although low-level expression is observed in cerebral cortex, hippocampus and striatum⁹. We found that GTRAP41 and GTRAP48 were expressed predominately in the cerebellum (Fig. 1e–g), with low-level immunoreactivity in striatum, hippocampus and thalamus. Immunofluorescence microscopy revealed that all three proteins are expressed in cerebellar Purkinje cell soma and dendrites, with little axonal staining (Fig. 1h–m).

We first confirmed the biochemical interaction between GTRAP41/GTRAP48 and EAAT4 by an *in vitro* binding assay. Full-length Myc-tagged GTRAP41 and GTRAP48 were expressed in HEK 293T cells. The solubilized cell extracts were then mixed with bead-linked glutathione S-transferase (GST)–EAAT4 or GST alone, and the bound proteins were eluted. GTRAP41 and GTRAP48 were retained specifically by the GST–EAAT4 fusion protein, but not by GST alone (Fig. 2a). A stable, rat EAAT4-expressing cell line was generated in HEK 293T cells (HEK-rEAAT4) and transfected with cDNAs encoding Myc-tagged GTRAP41 and GTRAP48. We used antibodies directed at the amino terminus of EAAT4 to immunoprecipitate the antigen plus any associated protein. Western blot analysis using an anti-c-Myc antibody showed that GTRAP41 and GTRAP48 co-immunoprecipitated with EAAT4 (Fig. 2b).

Similarly, when the anti-c-Myc antibody was used, EAAT4 was co-immunoprecipitated with GTRAP41 and GTRAP48 (Fig. 2c). *In vivo*, GTRAP41 (Fig. 2d) and GTRAP48 (Fig. 2f) were co-immunoprecipitated with EAAT4 from brain by antibodies directed at the N terminus of EAAT4 but not by antibodies directed at the C terminus (Fig. 2d, f). As the site of interaction is within the C terminus of EAAT4, however, it is likely that the C-terminal antibodies disrupt the protein–protein interaction. Furthermore, GTRAP41 and GTRAP48 seem to interact specifically with EAAT4, as we could not co-immunoprecipitate GTRAP41 and GTRAP48 from brain with antibodies directed at other glutamate transporters (Fig. 2d, f).

Western blot analysis confirmed that the immunoprecipitating antibodies pulled down their corresponding antigen (see Supplementary Information). No co-immunoprecipitation was observed when the precipitating antibody was omitted or pre-absorbed (Fig. 2b, e, g). We next determined the region of EAAT4 that binds GTRAP41 and GTRAP48. We used a series of successively larger C-terminal deletions of the original, 77-amino-acid, C-terminal EAAT4 bait in a yeast two-hybrid screen. Residues 555–561 and 527–534 appear to be required for GTRAP41 and GTRAP48 binding, respectively.

As GTRAP48 possesses areas of homology to p115 and PDZ-Rho-GEF, two RhoGEFs that selectively activate Rho^{16,17}, we investigated whether GTRAP48 interacts with the Rho family of GTPases. We measured the amount of GTP- γ S that bound to GST–RhoA, GST–Cdc42 and GST–Rac in the presence of full-length GTRAP48 or p115, and found that GTRAP48, like p115, shows a specific guanine nucleotide exchange activity for Rho (Fig. 3a, b). Co-immunoprecipitation assays also showed that GTRAP48 interacts with the active form (in the presence of aluminium fluoride) but not the inactive form of the G α_{13} subunit and therefore, may act as a link between G-protein-coupled receptors and their downstream targets (Fig. 3c). Specificity of the G α_{13} antibody has been previously described¹⁸.

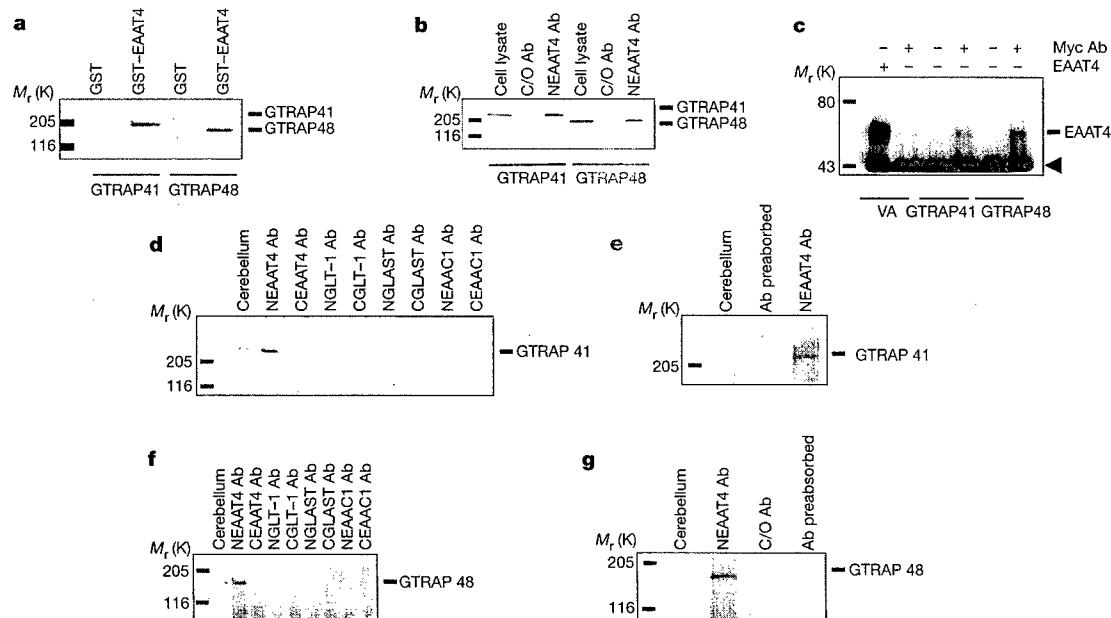


Figure 2 Interaction of GTRAP41 and GTRAP48 with EAAT4. **a**, Binding of Myc–GTRAP41 and Myc–GTRAP48 to GST–EAAT4. **b, c**, GTRAP41, GTRAP48 or vector alone (VA) were expressed (as indicated by the bars) in HEK-rEAAT4 cells. Immunoprecipitations were performed either with the N-terminal anti-EAAT4 antibody (**b**), or with the antibodies (Ab) indicated above the lanes (**c**). Immunoprecipitates were analysed by immunoblotting using anti-c-Myc (**b**) or C-terminal anti-EAAT4 (**c**); arrowhead indicates protein-A–

Sepharose band) antibodies. **d–g**, Extracts of rat brain were immunoprecipitated with antibodies (**d, f**) directed at the N terminus and C terminus of the glutamate transporters (as indicated above the lanes), no antibody or antibody pre-absorbed with peptide (**e, g**). Immunoblots were probed with the anti-GTRAP41 (**d, e**) and the anti-GTRAP48 (**f, g**) antibodies.

Rho regulates the remodelling of the actin cytoskeleton through various actin-binding proteins, although the mechanism is not well characterized¹⁹. As GTRAP48 can activate Rho, we next determined whether the expression of GTRAP48 induced reorganization of the actin cytoskeleton and whether it altered the distribution of GTRAP41, a possible actin-binding protein. When GTRAP41 was expressed alone there was a close relationship between actin and GTRAP41 at the cell membrane, but there were very few organized

actin filaments (Fig. 3d, e). Conversely, when GTRAP41 and GTRAP48 were co-expressed, GTRAP41 colocalized with actin in structures that resembled actin-stress fibres (Fig. 3f, g), a typical Rho-dependent effect. We noted that the overexpression of GTRAP48 also induced the formation of membrane ruffling and filopodia (Fig. 3h, i), suggesting some degree of cross-talk between the small GTPases, as these are typical Rac- and Cdc42-dependent effects.

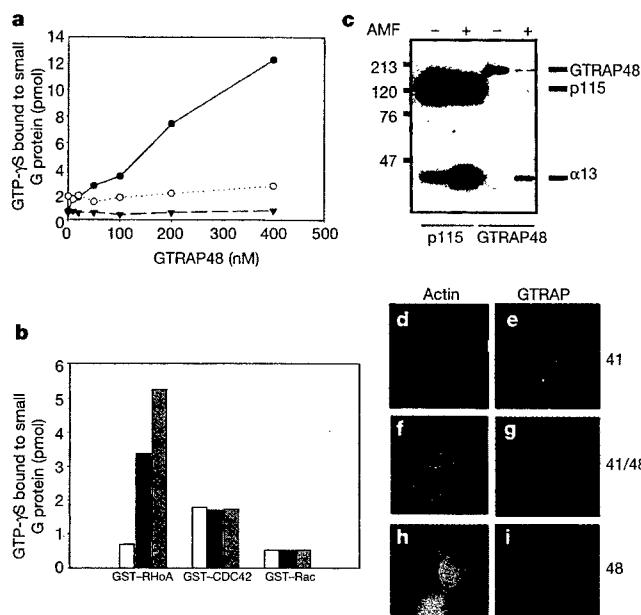


Figure 3 Guanine nucleotide exchange activity of GTRAP48. **a**, Binding of GTP- γ S to GST-RhoA (filled circles), GST-CDC42 (open circles) and GST-Rac (filled triangles) was measured after 10 min at 30 °C in the presence of the indicated concentrations of full-length GTRAP48 as described in Methods. **b**, Binding of GTP- γ S to the indicated GTPases in either the absence (white), or the presence of 100 nM GTRAP48 (black) or 100 nM

p115-RhoGEF (grey). **c**, Binding of active G α ₁₃ to Glu-tagged (EE) GTRAP48 and p115 RhoGEF. **d–i**, HEK-rEAAT4 cells transfected with either vector alone (**d**, **e**), Myc-tagged GTRAP41 and GTRAP48 (**f**, **g**) or Myc-tagged GTRAP48 (**h**, **i**). Actin filaments were visualized with FITC- or rhodamine-conjugated phalloidin.

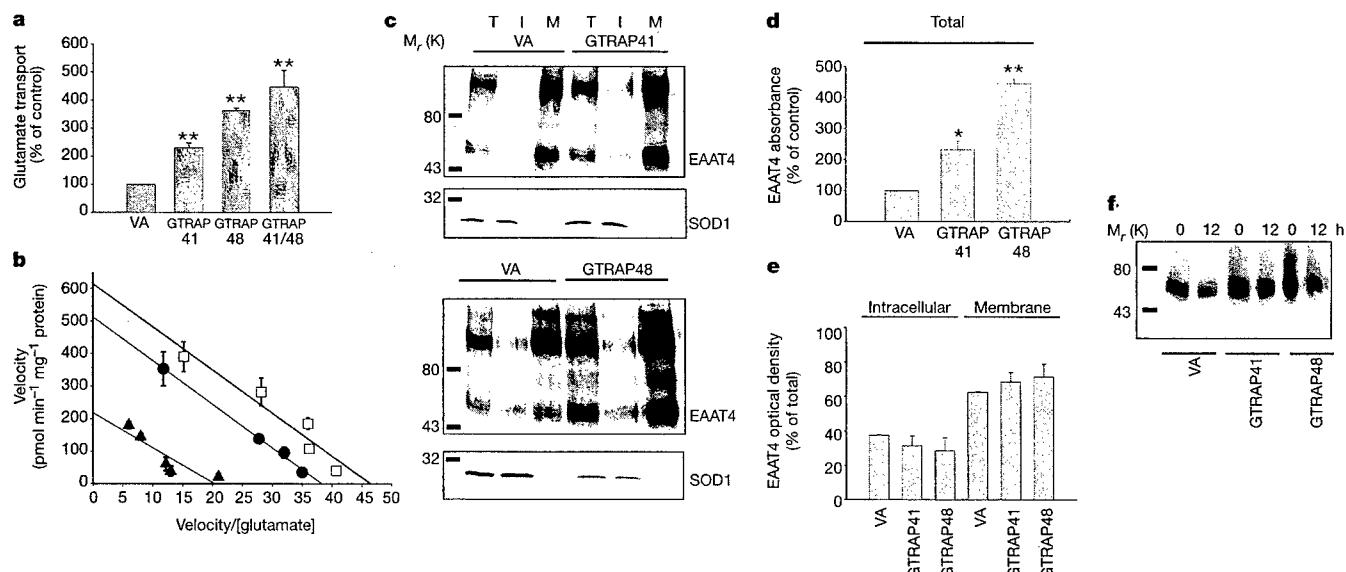


Figure 4 Effect of GTRAP41 and GTRAP48 on Na⁺-dependent [³H]L-glutamate uptake. **a**, GTRAP41 and GTRAP48 expression increased glutamate uptake (5 μ M) significantly over cells transfected with vector alone (VA; $n = 4$; ** $P < 0.005$). **b**, Kinetic data showed that GTRAP41 (open squares) increased the V_{max} from 220 to 605 pmol mg^{-1} min⁻¹ and increased the K_m slightly from 7 to 11 μ M, as compared with EAAT4 alone (filled triangles). GTRAP48 increased the V_{max} from 208 to 512 pmol mg^{-1} min⁻¹ (filled circles) and

increased the K_m from 10 to 13 μ M. **c**, Immunoblots of total (T), intracellular (I), and biotinylated fractions (M) of HEK-rEAAT4 cells transfected with GTRAP41 and GTRAP48. **d**, Quantitation of immunoblots for total EAAT4 protein ($n = 3$; ** $P < 0.005$, * $P < 0.05$). **e**, Ratio of membrane-bound to intracellular EAAT4. **f**, Cells transfected with GTRAP41, GTRAP48 or vector alone incubated with cycloheximide (10 μ g ml⁻¹) for 12 h.

We determined whether there was a functional association between GTRAP41, GTRAP48 and EAAT4. We measured the sodium-dependent glutamate transport activity of HEK-rEAAT4 cells that had been transfected with one or both of the interacting proteins. GTRAP41 and GTRAP48 produced respective twofold and fourfold increases in glutamate transport (Fig. 4a), and their co-expression resulted in a further increase in glutamate uptake. Kinetic analysis indicated that GTRAP41 and GTRAP48 produced an increase in the V_{max} of glutamate transport activity (Fig. 4b). GTRAP41 and GTRAP48 may therefore enhance glutamate transport either through an increase in the catalytic rate of the transporter or through an increase in cell-surface availability.

To examine changes in the cell-surface levels of EAAT4, we used a cell-membrane-impermeant biotinylation reagent to label cell-surface proteins selectively. Figure 4c shows western analysis of a representative biotinylation experiment for GTRAP41 and GTRAP48. The total amount of EAAT4 increased when GTRAP41 and GTRAP48 were co-expressed (Fig. 4d); in contrast, the total amount of SOD1, a control for the total amount of protein loaded, was unaltered or decreased in the GTRAP41 or GTRAP48 samples, respectively. The percentage of total EAAT4 that was biotinylated remained the same when GTRAP41 and GTRAP48 were co-expressed, showing that most of the increase in total EAAT4 protein was located at the cell surface and not in an intracellular

pool (Fig. 4e).

These results indicated that GTRAP41 and GTRAP48 stabilize and/or anchor EAAT4 at the cell membrane, making it less likely to be internalized and subsequently degraded; however, we could not rule out the possibility of increased expression of the cell's native gene. To address this issue, we treated cells with cycloheximide, an inhibitor of protein synthesis, 48 h after transfection (Fig. 4f). Densitometry showed that 12 h after treatment the EAAT4 protein in HEK-rEAAT4 cells was reduced to $54 \pm 0.6\%$ of its level before cycloheximide treatment. In contrast, $81 \pm 2\%$ and $74 \pm 1.7\%$ of the EAAT4 protein remained after 12 h when GTRAP41 and GTRAP48 were co-expressed, respectively. These results provide compelling evidence that GTRAP41 and GTRAP48 do stabilize EAAT4 at the membrane.

To determine whether the EAAT4/GTRAP48 interaction is required to mediate the increase in EAAT4 activity, we transfected HEK-rEAAT4 cells with GTRAP48 constructs lacking the last 155 amino acids, which were pulled out by EAAT4 in the yeast two-hybrid screen. The C-terminally truncated GTRAP48 had only a modest effect on stimulating EAAT4 activity, indicating that the protein-protein interaction is responsible for most of the increase in uptake activity (Fig. 5a). We also co-transfected HEK-rEAAT4 cells with GTRAP48 and a Myc-tagged cDNA construct encoding the last 77 amino acids of EAAT4, to disrupt the interaction of GTRAP48 with full-length EAAT4. We found that the co-expression of this construct inhibited the GTRAP48-mediated effect by about 25%, but co-expression of a smaller construct (residues 1,452–1,578), lacking the GTRAP48-binding domain, had no effect (Fig. 5b). Together, these results indicate that the EAAT4/GTRAP48 interaction is important in modulating EAAT4 uptake activity.

The physiological relevance of GTRAP41 and GTRAP48 on EAAT4 uptake activity *in vivo* was subsequently examined by the intra-cisternal injection of HSV amplicon vectors expressing GTRAP41 and GTRAP48. Cerebellar glutamate uptake was measured 48 h after injection, and was elevated when GTRAP41 and GTRAP48 were expressed but not when the control HSVlac amplicon vector was injected (Fig. 5c). Dihydrokainic acid (DHK), an inhibitor of GLT-1-mediated glutamate transport, had no effect on cerebellar glutamate uptake, ruling out any involvement of GLT-1. There is no method to distinguish functionally between GLAST, EAAC1 and EAAT4; but, as GTRAP41 and GTRAP48 do not interact directly with any other transporter, it is likely that the observed increase in uptake is due to an increase in EAAT4 activity. Western blot analysis confirmed an increased expression of GTRAP41 and GTRAP48 in the cerebellum after the injections. Preliminary studies of primary cultures of rat Purkinje cells indicate that EAAT4 and GTRAP41 may colocalize perisynaptically at 70% of synapses.

Glutamate transporters, through their rapid and efficient removal of glutamate from the synaptic cleft, are critical in glutamatergic plasticity and the prevention of glutamate-mediated excitotoxicity. Identifying and characterizing the glutamate transporter associated proteins GTRAP41 and GTRAP48 has begun to unravel the complex mechanism of glutamate uptake. In the modulation of EAAT4 glutamate transport, our findings implicate a role for G-protein signalling, a pathway that may involve Rho activation, and anchoring to the actin cytoskeleton (Fig. 5d). These proteins may also modulate the perisynaptic distribution of EAAT4 at glutamatergic synapses. Future studies are required to delineate the signalling pathway of GTRAP41 and GTRAP48, and how their interaction may be relevant to normal and abnormal glutamatergic neurotransmission, as altered EAAT4 function may be involved in the neurodegenerative disease spinocerebellar atrophy (SCA1)²⁰. By understanding the physiological regulation of the EAAT4 transporter, we may be able to identify possible therapies for SCA1 or other toxic insults that lead to the degeneration of Purkinje cell neurons.

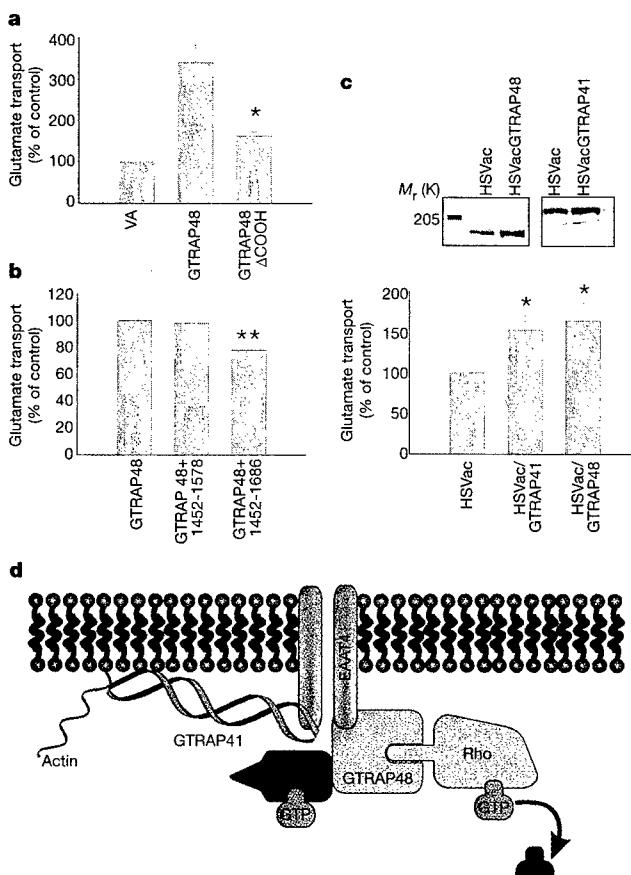


Figure 5 Modulation of EAAT4 activity. **a**, GTRAP48 Δ COOH results in a very small increase in EAAT4 uptake activity compared with full-length GTRAP48 ($P < 0.05$). **b**, Overexpression of the EAAT4 C-terminus reduced the GTRAP48-mediated effect on EAAT4 activity ($**P < 0.005$). **c**, Intra-cisternal injection of HSVlac amplicons expressing GTRAP41 and GTRAP48 increased glutamate uptake *in vivo* ($n = 6$; $*P < 0.05$). Western blot analysis demonstrates increased expression of GTRAP41 and GTRAP48. **d**, A possible model of the coupling of EAAT4 to a Rho GTPase signal transduction cascade and to the actin cytoskeleton via GTRAP48 and GTRAP41. GDP, guanosine diphosphate; GTP, guanosine triphosphate.

Methods

Yeast two-hybrid screen

We screened a rat brain cDNA library (Clontech) using the final 77 amino acids of EAAT4 as bait (pGBT9). For the EAAT4 C-terminal domain analysis, different regions of the final 77 amino acids of EAAT4 were subcloned in-frame into the pGBT9 vector.

Cloning of full-length GTRAP41 and GTRAP48 cDNAs

Marathon cDNA amplification (CLONTECH) was used to perform both 5'- and 3'-RACE on cDNA synthesized from rat brain poly(A)⁺ RNA.

Antibodies

Affinity-purified polyclonal antisera to EAAT4, GTRAP41 and GTRAP48 were generated as described⁶. We synthesized peptides corresponding to epitopes of EAAT4 (C-terminal, EKGASRGRGGNESA; N-terminal, KNSLFLRESGAGGGCL), rat GTRAP41 (KRGAPSPMPQSRSSE) and rat GTRAP48 (KTPERTSPSHRQPSD). The anti-c-Myc monoclonal antibody was from Boehringer Mannheim. For visualization of intracellular F-actin organization, the cells were probed with Texas-red-conjugated or fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma).

Transfection and maintenance of HEK-rEAAT4 cells

The EAAT4 cDNA was subcloned into the pcDNA3.1/Hygro(+) (Invitrogen) using the EcoRI restriction site. The plasmid was linearized with SspI and transfected into HEK 293T cells. Forty-eight hours after transfection, the cells were split to 50% confluence, and hygro-Mycin (Invitrogen) was added at a concentration of 50 µg ml⁻¹. After about 2–3 weeks of selection, a serial dilution was carried out and cells were plated out, without selection, in a 96-well plate to obtain one cell per well. Several colonies were picked, expanded in selective medium and checked for expression by western blotting.

Fusion proteins and *in vitro* binding

Full-length EAAT4 was subcloned into the EcoRI site of the GST-fusion vector pGEX-6P-1 (Pharmacia). Synthesis of recombinant proteins in BL21 cells (Novagen) was induced by 0.1 mM isopropyl β-D-thiogalactoside for 2 h at 30 °C and purified according to the protocol provided by Pharmacia. HEK 293T cells were transfected with Myc-tagged GTRAP41 or GTRAP48 and gathered in ice-cold immunoprecipitation (IP) buffer. The cellular lysate was incubated with GST or GST-EAAT4 immobilized on glutathione-Sepharose-4B, and washed to remove nonspecifically bound proteins. Specifically bound proteins were eluted with 2× SDS loading buffer and analysed by immunoblotting using an anti-c-Myc antibody.

Co-immunoprecipitation

Full-length GTRAP41 and GTRAP48 cDNAs were subcloned into the NotI site of a Myc-tagged pRK5 vector and used to transfect HEK-rEAAT4 cells. After transfection (48–72 h), cells were solubilized with 1 ml of ice-cold IP buffer for 2 h at 4 °C with rotation and centrifuged to remove cellular debris. Antibody was added to 0.5 ml of supernatant and incubated overnight at 4 °C. We dissected and prepared the cerebellum from a Sprague-Dawley rat as described²¹. For each immunoprecipitation, 500 µg of the Triton-lysate was incubated overnight at 4 °C with 5 µg of antibody. Protein-A-Sepharose (Pharmacia) was then added for 2 h at 4 °C, washed once with IP buffer and three times with IP buffer minus Triton X-100. Bound protein was eluted by boiling in 2× SDS loading buffer, and analysed by immunoblotting.

Immunohistochemistry

Rat brain sections were stained as described⁹, using the following antibodies: C-terminal anti-EAAT4 (1.5 µg ml⁻¹), anti-GTRAP41 (127 ng ml⁻¹) or anti-GTRAP48 (132 ng ml⁻¹) antibodies. Texas-red and FITC-conjugated secondary antibodies were used at dilutions of 1:200.

Guanine nucleotide exchange assay

Small G proteins GST-RhoA, GST-CDC42 and GST-Rac were expressed in bacterial cells and affinity purified to ~80% purity using a glutathione column. We incubated 20 pmoles of each protein with 100 pmoles GTP-γS for 10 min at 30 °C with varying concentrations of full-length GTRAP48 or p115. The binding reactions were filtered through BA-85 nitrocellulose and the amount of GTP-γS bound to small G protein was quantified by scintillation counting of the dried filters. Binding of Gα₁₃ to GTRAP48 was assayed as described¹⁸.

Na⁺-dependent glutamate transport activity

HEK-rEAAT4 cells transfected with GTRAP41 and GTRAP48 were grown in a monolayer on six-well plates, and assays were conducted 72 h after transfection as described²². We subcloned GTRAP41 and GTRAP48 into the EcoRI site of HSV-PrPUC amplicon parent vector²³. Amplicon vector DNA (3.6 µg) and pBAC-V2 DNA (25 µg) were used to transfet 2×10⁷ baby hamster kidney cells as described²⁴. Virus was collected 72 h after transfection, and titred as described²⁵. Expression particles (2×10⁵) were injected intracisternally into male Sprague-Dawley rats (250 g) obtained from Zivic Miller. Forty-eight hours after injection the rats were killed, synaptosomal preparations of the cerebelli were prepared using a polytron, and glutamate uptake was measured.

Biotinylation

Biotinylation of cell-surface proteins was done as described²⁶. We used SOD1 to control for total protein and to determine whether the biotinylation reagent labels proteins in the intracellular compartment.

Statistics

Statistical differences were determined by Student's *t*-test for two-group comparisons.

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